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**Chemistry**

# Exploring ADMET properties and the anticancer potential mechanism of a new organoselenium compound using network pharmacology and *in vitro* study

## Explorando as propriedades ADMET e potenciais mecanismos anticancerígenos de um novo organoseleneto utilizando farmacologia de rede e estudo de atividade antitumoral *in vitro*

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## ABSTRACT

Complex genetic mutations and malignant transformations in cancers have plagued the world. Modified variants of nucleoside analogs have proven to be allies in the hope of promising new treatments in anticancer therapy. However, it sometimes faces difficulties related to its low bioavailability and resistance mechanisms. In this study, investigative and initial in silico and in vitro analyses were performed on a new organoselenium compound, 5-Se-(phenyl)-3-(ferulic-amido)-thymidine (AFAT-Se). Different *in silico* platforms were used to explore the ADMET properties and possible pharmacological and toxicological effects of AFAT-Se, and its anticancer potential was assessed by *in vitro* studies. AFAT-Se complied with Lipinski's rules, exhibiting favorable pharmacokinetic properties, interaction with common drug metabolic enzymes, toxicities that require further study, and cytotoxicity toward the HT-29 tumor cell line, as evidence of its potential as an antineoplastic agent. Therefore, critical molecular targets were identified in cancer-related pathways; thus, the organoselenium AFAT-Se is a promising candidate for further studies on this pathology.

**Keywords**: Pharmacokinetic properties; Cell culture; Targets

## RESUMO

Mutações genéticas complexas e transformações malignas são muito comuns em células tumorais. Variantes modificadas de análogos de nucleosídeos têm se mostrado uma alternativa promissora

na busca por novos tratamentos na terapia anticancerígena. No entanto, esses compostos às vezes apresentam dificuldades relacionadas à sua baixa biodisponibilidade e resistência do tecido tumoral a esses. Neste estudo, foram realizadas análises investigativas iniciais *in silico* e *in vitro* em um novo organoseleneto, 5-Se-(fenil)-3-(ferulico-amido)-timidina (AFAT-Se). Diferentes plataformas *in silico* foram usadas para explorar as propriedades ADMET e os possíveis efeitos farmacológicos e toxicológicos do AFAT-Se, e seu potencial antitumoral foi avaliado por estudos *in vitro*. O AFAT-Se cumpriu as regras de Lipinski, exibindo propriedades farmacocinéticas favoráveis, interação com enzimas metabólicas comuns de medicamentos, toxicidades que requerem mais estudos e citotoxicidade contra a linhagem celular tumoral HT-29, evidenciando seu potencial como agente antineoplásico. Portanto, foram identificados alvos moleculares críticos em vias relacionadas ao câncer; sugerindo que o organoseleneto AFAT-Se é uma promissora alternativa para estudos futuros nesta patologia.

**Palavras-chave**: Propriedades farmacocinéticas; Cultivo celular; Alvos

## 1 INTRODUCTION

Cancer is a group of diseases that causes public health problems worldwide (Siegel et al., 2023). The carcinogenesis process involves several genetic changes that may lead to malignant derivatives with autonomy concerning growth signals, unfeeling of growth suppressive signals, resistance to apoptotic cell death, replicative potential, angiogenesis, and invasive and metastatic capability. Several chemotherapeutics have been used to control these diseases, affecting critical cell division processes. However, despite the numerous treatment options, the difficulties faced by this class of drugs still limit the search for new antitumor agents (Shields, 2017; Steinbrueck et al., 2020).

Nucleoside analogs act in significant numbers in antitumor treatment. This class of antimetabolites, especially modified ones, can disrupt metabolic and regulatory pathways through their resemblance to purine and pyrimidine nucleosides. Possible nucleoside transporters and phosphorylation uptake can lead to interference in DNA/RNA synthesis and repair. Capecitabine, gemcitabine, clofarabine, and cytarabine are examples of this class, which have been approved as anticancer agents, in addition to promising studies involving these antimetabolites. However, problems such as low absorption, low conversion to an active triphosphate metabolite, degradation,

clearance, and even tissue resistance remain evident, making the research and promotion of new compounds of this class a focus of attention (Guinan et al., 2020; Macedo et al., 2022).

In discovering and optimizing biomolecules, knowledge about their safety, efficacy, toxicity, and pharmacokinetics is essential to determine their effectiveness and therapeutic success (Pires et al., 2015; Pires et al., 2018). To reduce the risks in the last stage of drug development and give greater importance to promising compounds, *in silico* computational methods and even *in vitro* approaches can be great allies. Both have many features that allow the estimation of the properties of the molecules under study (Fatima et al., 2020).

In this study, a new organoselenium nucleoside analog, 5-Se-(phenyl)-3-(ferulic-amido)-thymidine (AFAT-Se), was analyzed for ADMET properties and possible pharmacological effects using network pharmacology method and an *in vitro* assay. First, the compound SMILE was obtained from the PubChem webserver. Lipinski's Rule of Five criteria was determined in the Molinspiration tool using the SMILE obtained. Furthermore, the ADMET compound properties were analyzed using the pkCSM, admetSAR, and SwissADME platforms. In addition, possible biological activities were predicted using the WAY2Drug web server, and cytotoxicity in cell lines was analyzed using the CLC-Pred platform. In addition, one of the indicated tumor cell lines, HT-29, was used to evaluate *in vitro* activity to confirm the potential activity of the organoselenium compound. Furthermore, genes that are potential molecular targets and Protein-Protein Interaction targets were predicted using the DIGEP-Pred, string, and Cytoscape platforms. Finally, GO and KEGG enrichment analyses were performed using the WebGestalt tool to identify the genes involved and their possible mechanisms of action. Therefore, this study contributes to the ongoing search for effective cancer therapies by investigating the antitumor potential of the novel organoselenium compound, AFAT-Se. Using both *in silico* and *in vitro* approaches is an interesting and effective way to identify critical molecular targets

within cancer-related pathways and provide a comprehensive assessment of AFAT-Se's ADMET properties and anticancer activity.

## 2 MATERIALS AND METHODS

### 2.1 Materials

2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), trypsin-EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA), fetal bovine serum (FBS), antibiotic solution (with 10,000 units penicillin and 10 mg streptomycin/mL), and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with L-glutamine (584 mg/l), were purchased from Sigma-Aldrich (São Paulo, SP, Brazil).

The organoselenium compound AFAT-Se was obtained from LabSelen-NanoBio (Federal University of Santa Maria, Brazil). This organoselenium was synthesized and characterized as previously described (Leal et al., 2022).

### 2.2 Obtaining the chemical structure and the SMILE in the PubChem database

PubChem (https://pubchem.ncbi.nlm.nih.gov/) is an open bank of chemical and bioactivity information that is fundamental as a data source in several areas. This web base is based on hundreds of databases, which are constantly growing. The structure of the organoselenium was made via the "Draw Structure" option to recover the SMILE [C@H]1(C(C[C@@H](O1)N2C=C(C(N(C2=O)[H])=O)C)N(C(C=CC3=CC(=C(C=C3)O[H])OC)=O)[H])C[Se]C4=CC=CC=C4 that allows the subsequent analyses.

### 2.3 Molecule analysis using Lipinski's Rule of Five criteria on the Molinspiration platform

Molinspiration (https://www.molinspiration.com/) is a platform with a variety of tools that allows the determination of the octanol-water partition coefficient (LogP)

through a robust method that involves the sum of fragment-based contributions and correction factors, thus enabling the practical verification of all organic molecules. It was also used to determine the total polar surface area (TPSA) of the molecule using the sum of fragment contributions process and simple molecular descriptors of the number of hydrogen bond donors (HBD) and acceptors (HBA), molecular weight (MW), number of atoms, and rotatable bonds (Nrotb) from the SMILE of the molecule of interest. Considering the contributions of these groups, the platform also evaluates the molecular volume.

### 2.4 Prediction of ADMET profile using pkCSM, admetSAR, and SwissADME web servers in combination

The webservers pkCSM - Predicting Small-Molecule Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures (https://biosig.lab.uq.edu.au/pkcsm/), admetSAR - ADMET structure-activity relationship database (http://lmmd.ecust.edu.cn/admetsar2), and SwissADME (http://www.swissadme.ch/) of Swiss Institute of Bioinformatics provide predictions about pharmacokinetic processes (Absorption, Distribution, Metabolism, Excretion and Toxicity) of drug candidates from their SMILE.

### 2.5 WAY2Drug to Predicted Biological Activity

WAY2Drug (http://www.way2drug.com/PASSOnline/index.php) is an informational-computational platform capable of predicting the biological activity of drug-like compounds. This web base uses more than 250,000 biologically active structures to predict the potential physical activity using the SMILE of the molecule. The viability of a particular spectrum of biological activity is given by Pa (probability “to be active”) and Pi (probability “to be inactive”). In this case, Pa > 0.7 was used. Notably, the values of Pa and Pi are more closely related to the similarity with the training set of the platform and usually have no direct relationship with the quantitative characteristics of the activity

### 2.6 CLC-Pred (Cell Line Cytotoxicity Predictor) to assess the cytotoxicity in cancer cell lines

This computer-based CLC-Pred (https://www.way2drug.com/clc-pred/) allows for *in silico* screening of compounds that may be biologically active with an average prediction accuracy of 93%. SMILE was inserted into the platform to analyze non-transformed and cancer cell lines. In this data base, Pa (probability "to be active") > Pi (probability "to be inactive") was used, and IAP (Invariant Accuracy of Prediction), which is the average accuracy of prediction, was evaluated. Similarly, the Pa and Pi values are related to the training data set.

### 2.7 Cell line and culture conditions

The HT-29 tumor cell line (human colon adenocarcinoma) was cultured in 75 $cm^2$ culture flasks containing DMEM medium (4.5 g/L glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 10,000 U/mL penicillin and 10 mg/mL streptomycin. The cells were maintained at 37°C under 5% $CO_2$ in an appropriate incubator until the exponential growth phase reached 80% confluence; then, the cells were collected with trypsin-EDTA and used in the assays.

### 2.8 *In vitro* antitumor activity using a two-dimensional monolayer (2D) assay

The 2D cell culture assay to verify the antineoplastic potential was performed with a cell density adjusted to 6,5 × $10^4$ cells/mL, which was seeded in a 96-well plate and incubated for 24 h at 37 °C in an atmosphere of 5% $CO_2$. The organoselenium concentration range used in the assay to verify growth inhibition was 0.1 - 40 µg/mL, diluted in DMEM with 5% FBS. After 72 h of incubation, cell viability was confirmed using an MTT assay. A MTT solution, 0.5 mg/mL concentration in DMEM without FBS, was added to each well and incubated for 3 h at 37°C under 5% $CO_2$. The medium was then replaced with 100 µL of DMSO to dissolve the purple formazan product, and the absorbance was measured at 550 nm using a microplate reader Multiskan FC (Thermo Fisher Scientific, Shanghai, China).

### 2.9 Screening of Gene Targets and Analysis of Protein-Protein Interaction

The SMILE of the molecule was loaded into DIGEP-Pred (http://www.way2drug.com/ge/) to identify the target proteins (downregulated proteins) using Pa > 0.7. Information regarding these proteins was obtained from the GeneCards database (https://www.genecards.org/). The STRING database (https://string-db.org/) was used to establish protein-protein interactions, providing an association network and filtering the data by high confidence score interactions and no more than 50 interactions in the first shell. Limiting the interactions to a maximum of 50 helps maintain a clear and manageable protein interaction network. Thus, only the most relevant interactions are retained, which minimizes the inclusion of indirect or unreliable connections that may not contribute to significant biological insights. In addition, the Cytoscape platform was used for a comprehensive network topology analysis.

### 2.10 Enrichment Analysis

Gene Ontology (GO) and KEEG pathways were performed using WebGestalt (https://www.webgestalt.org/). GO analysis involved defining targets containing molecular functions, biological pathways, and cellular components; the above 20 targets in these processes were considered the main targets. In addition, KEEG analysis facilitates the understanding of functionality at various levels.

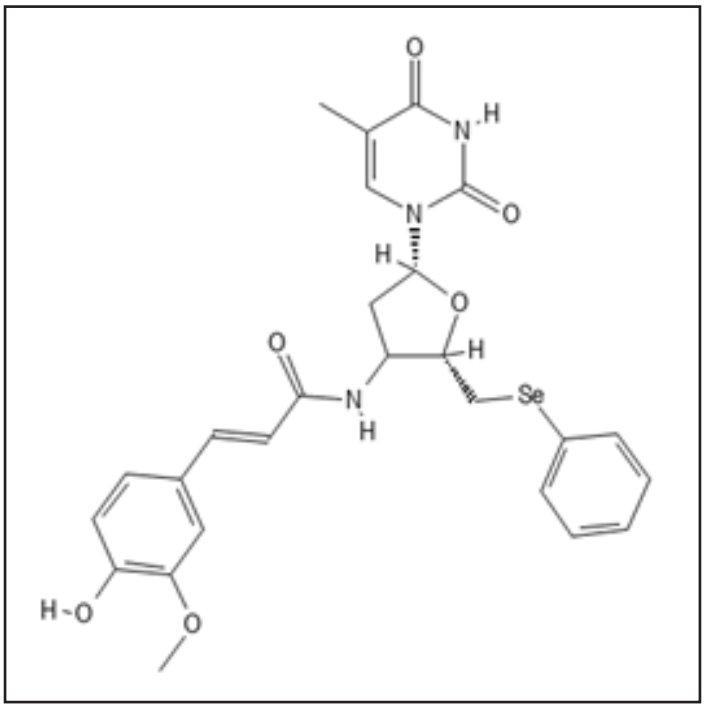
## 3 RESULTS

### 3.1 Initial information about the compound: Molecular Formula and Lipinski's Rule of Five

The design of the organoselenium molecule (Figure 1) was drawn in PubChem, and SMILE was obtained using its molecular structural formula. This allowed the analysis of Lipinski's rule of five and other parameters using the Molinspiration platform,

which evaluates rules that indicate whether a chemical compound could be drug-like with oral bioavailability. Therefore, a compound with drug-like properties must have specific criteria, which include less than 10 hydrogen bond acceptors and less than 5 hydrogen bond donors; molecular weight not greater than 500 g/mol, indicating hydrophobicity; logP, with a value below 5, not being able to violate more than one of these parameters (Lipinski, 2004). Additionally, to consider the permeability and flexibility, the polar surface area must be equal to or less than 140 and have no more than 10 rotational bonds, in that order (Veber et al., 2002). Still, the molecular volume is the feature that can determine factors such as intestinal absorption and blood-brain barrier penetration.

Figure 1 – The molecular structural formula of the organoselenium compound




Source: PubChem

The organoselenium compound subtly violated only one Lipinski rule, showing a molecular weight of 556.48 g/mol. As for the other parameters, the values aligned with what is expected from a drug with good bioavailability (Table 1).

Table 1 – Parameters measured in Molinspiration plataform

| Parameter | Organoselenium |
|---|---|
| Hydrogen bond acceptor (HBA) | 9 |
| Hydrogen bond donor (HBD) | 3 |
| Molecular Weight (MW) | 556.48 g/mol |
| Logarithm Octanol/water partition coefficient (LogP) | 2.97 |
| Polar Surface Area (TPSA) | 122.66 Å |
| Number of rotatable bonds (Nrotb) | 8 |
| Molecular Volume | 449.83 |

Source: Organized by the authors (2024), with data obtained by the Molinspiration platform

### 3.2 Prediction of ADMET Properties

Concerning the absorption parameters, the human intestinal absorption factor was above 30% on both platforms, 61.28% for pkCSM, and 87.26% for admetSAR. Promising percentages, such as values lower than 30%, indicate that this molecule is poorly absorbed. Similarly, the swissADME platform classifies the molecule as “high” in the gastrointestinal absorption parameter. The factor involving P-glycoprotein denotes that the organoselenium is an inhibitor of this efflux pump on the pkCSM and admetSAR platforms. However, the data differed when examining the P-glycoprotein substrate results, where both pkCSM and SwissADME platforms indicated that the compound would be a substrate for this factor, while the admetSAR software showed negative results for this condition.

In the distribution factors, such as permeability in the blood-brain barrier (BBB), the compound demonstrated values that would be poorly distributed to the brain in all cases. In particular, in the pkCSM software, it is also possible to verify the volume of distribution, which is intermediate at 1.13 L/kg, indicating an average distribution between the tissue and plasma. On this platform, the value of the fraction not bound to serum proteins (Fu) of 0.027 stands out, and it is classified as low (<0.05), which can impair the compound’s ability to cross cell membranes or diffuse. In agreement

with pkCSM, in the admetSAR web-based, the value for plasma protein binding of the organoselenium was 81.9%.

These platforms consider the Cytochrome P450 superfamily (CYP) to assess the metabolism responsible for the structurally different metabolization of xenobiotics (Esteves et al., 2021). The admetSAR and pkCSM tools identified the possibility that the molecule was a substrate for CYP2D6 and CYP3A4. In both cases, the organoselenium is not a substrate of the first but a substrate of the second. Metabolism was also evaluated by all platforms regarding the inhibition of CYP isoforms. According to the web-based method, the compound did not inhibit CYP1A2, CYP2C19, and CYP2D6 isoforms. Although, about CYP2C9 and CYP3A4, there were differences in the reading. The admetSAR and SwissADME platforms indicated that the organoselenium would not be a CYP2C9 inhibitor, while pkCSM showed that it would be. In addition, with the CYP3A4 isoform, pkCSM and SwissADME warn about the possibility of inhibition, while admetSAR does not offer this response.

The excretion is measured through parameters involving the Organic Cation Transporter 2 (OCT2) and total clearance. OCT2 is vital for renal elimination and clearance and mediates the initial step of renal secretion. The pkCSM tool indicated that the compound would not be a substrate for this system; in addition, in admetSAR, the result shows that the molecule would be an inhibitor of this transporter. The hepatic and renal clearance of the compound was evaluated in the pkCSM web-based through the total clearance, a factor related to the bioavailability of the dosage rate to reach the concentrations necessary for the action. The value found for the organoselenium under analysis was 2.73 log mL/min/kg (526.04 mL/min/kg).

The toxicity parameters are diverse. The maximum tolerated dose for humans (MTD) by the pkCSM tool is -0.417 log mg/kg/day (1.58 mg/kg/dia), which is the value that estimates the toxic dose limit of chemicals for human application. The pkCSM and admetSAR databases analyze a parameter inhibiting potassium channels encoded by human ether-a-go-gene (hERG), which is mainly associated with cardiac

toxicity (Garrido et al., 2020). In the first tool, which distinguishes hERG I and II, inhibition of hERG II is indicated. Furthermore, hERG inhibition is generally indicated by the admetSAR. However, there is evidence that hERG channels are significantly expressed in cancer cell lines, delimiting processes such as cell proliferation, apoptosis, angiogenesis, and migration, making it possible to consider it as a potential therapeutic target (He et al., 2020).

Another point evaluated by the two tools is the Ames test, a method that delimits the possibility of mutagenicity caused by the compound, and both indicated negative results; therefore, the organoselenium would possibly not act as a carcinogen (Zeiger et al., 2019). Furthermore, Oral Rat Acute Toxicity analysis was performed on both platforms, considering the lethal dose values (LD50) against rats. In the pkCSM tool, the value found was 2.558 mol/kg (1430.8 g/kg), and in the admetSAR platform, the compound was classified as grade III, considered moderate (Gomes et al., 2020). In addition, the value of Oral Rat Chronic Toxicity was evaluated only by the pkCSM tool, which was able to identify the lowest dose of the compound that induces adverse effects (LOAEL), which was determined to be 3.164 log mg/kg bw/day (1394.6 mg/kg bw/day).

The hepatotoxicity and skin sensitization parameters were also examined using the pkCSM and admetSAR platforms. In consensus by the two tools, organoselenium would be detrimental to normal liver function and cannot induce skin sensitization. However, analysis of nephrotoxicity using the admetSAR tool indicated that the compound could not generate this damage. Furthermore, the protozoan Tetrahymena pyriformis, a unicellular organism used in the fields of toxicology and ecotoxicology that has been used repeatedly as a toxic endpoint over the years, was also analyzed on both platforms, indicating this type of toxicity (Yu, 2020). In addition, when evaluating minnow toxicity, a parameter that takes ecotoxicology into account, the pkCSM platform indicates the occurrence of high toxicity with a value of 0.2 mM (< 0.5 mM), and the admetSAR tool corroborates with a positive result for fish aquatic toxicity (Wang & Chen, 2020).

### 3.3 WAY2Drug to Predicted Biological Activity

The Way2Drug Pass Online tool was used to predict the spectrum of biological activity. The natural potential of a drug-like molecule was given in probability on this platform. The first physical activity evidenced by the web base was antineoplastic (Pa = 0.839, Pi = 0.008). Second, the platform showed a factor that plays a central role in the cellular stress response, such as DNA damage repair, cell cycle arrest, apoptosis, and senescence; that is, the organoselenium would be a TP53 expression enhancer (Pa = 0.835, Pi = 0.008). Then, directly linked to the first factor, it appears to have anticarcinogenic activity (Pa = 0.805, Pi = 0.005). Lastly, in agreement with other biological activities, Way2drug indicated that the molecule had chemoprotective activity (Pa = 0.731, Pi = 0.003).

### 3.4 Results of CLC-Pred (Cell Line Cytotoxicity Predictor)

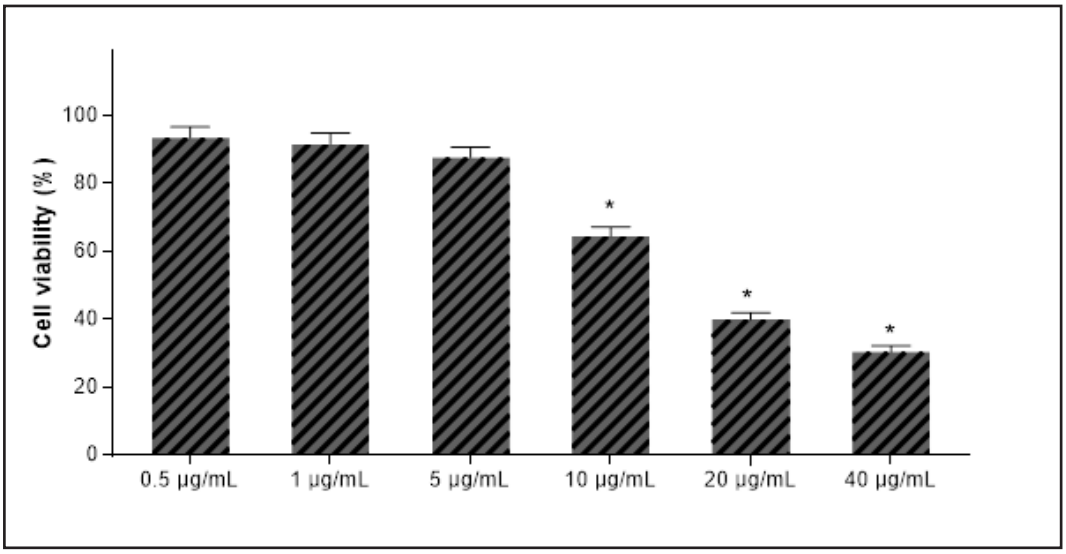
Cytotoxicity in cell lines and tissues/organs was predicted using a known chemoinformatics CLC-Pred tool. The platform indicated fifty-five different cell lines, with an average prediction accuracy of 0.802 - 1.000 for the compound under study.

Cytotoxicity was observed in different types of cancer cells and was associated with other organs. Indeed, it has been identified to be toxic to adenocarcinoma, carcinoma, leukemia, hepatoblastoma, glioblastoma, lymphoma, sarcoma, and melanoma cells.

### 3.5 *In vitro* antitumor activity assay

Cytotoxicity was evaluated using one of the possible cancer cell lines presented in the CLC-Pred Tool, the human colorectal adenocarcinoma cell line (HT-29). The results (Figure 2) showed a significant drop in cell viability starting at a concentration of 10 µg/mL, which highlights the promising application of organoselenium as an anti-tumor drug.

Figure 2 – *In vitro* cell viability by MTT assay in HT-29 cell line after 72 h of treatment. Data are expressed as the mean of three independent experiments ± SE. Statistical analyses were performed using ANOVA. * Indicates significant difference from the control cells ($p < 0.05$)




Source: Authors (2024)

### 3.6 Prediction of the Organoselenium's Biological Targets

The potential targets of the compound, which are directly related to its mechanism of action, were obtained using DIGEP-Pred (Table 2). These targets were downregulated by organoselenium and used for further analyses in this study.

CHEK1 is a protein-coding gene, part of the serine/threonine kinase family, and is involved in cell cycle control, damage response, and DNA checkpoints. This gene has two identified subtypes: Chk1 and Chk2. Thus, downregulation of this gene in cancer cells can lead to defects in G2 and SM checkpoints responsible for premature entry into mitosis, resulting in cell death. In addition, cancer cells use this activation system for DNA repair and, consequently, for more remarkable survival; thus, its inactivation may be an essential anticancer mechanism. Another critical factor in the antitumor analysis is that the inhibition of Chk1 may suppress P-gp levels, which would decrease the efflux of the molecule (Ahmed et al., 2022).

Table 2 – Information about the target genes of the organoselenium compound from the GeneCards database

| Gene ID | Symbol | Name |
|---|---|---|
| 1111 | CHEK1 | Checkpoint kinase 1 |
| 3952 | LEP | Leptin |
| 9370 | ADIPOQ | Adiponectin, C1Q, and collagen domain-containing |

Source: Organized by the authors (2024), with data obtained by the GeneCards database

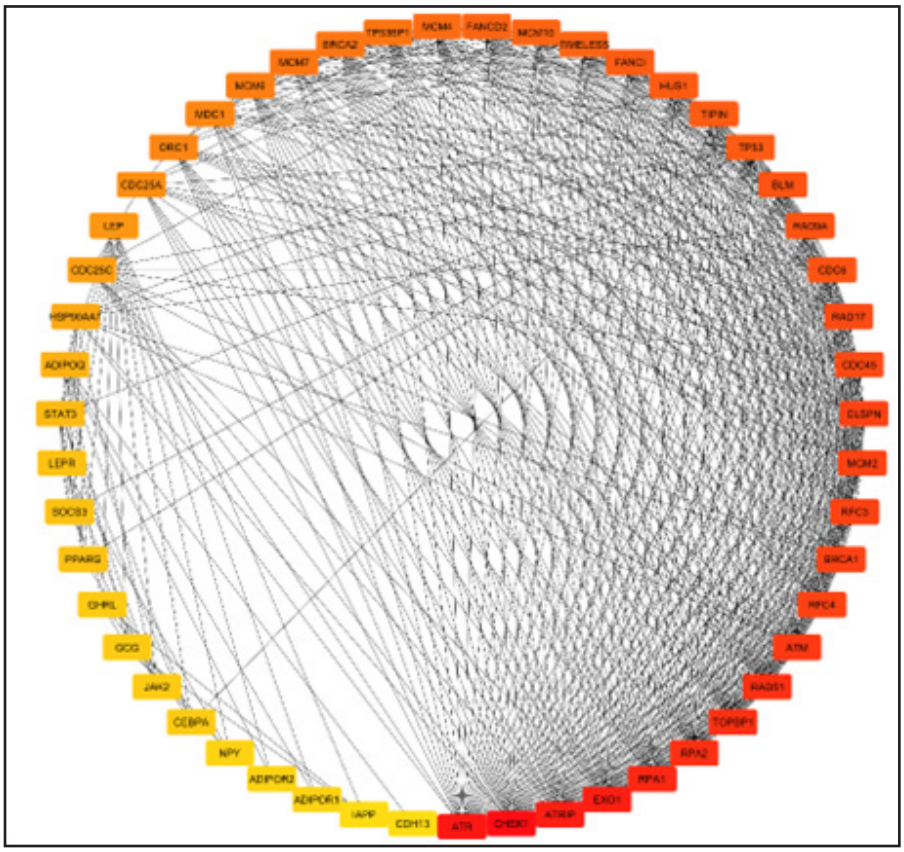
LEP encodes a glycosylated protein released mainly by adipocytes and in reduced amounts by the stomach, skeletal tissue, mammary gland, heart, kidney, brain, and placenta. It interferes with energy homeostasis, body weight, immunity, reproduction, hematopoiesis, and bone metabolism. Therefore, it is worth mentioning that this system is related to several factors that favor tumors. Hence, its reduced regulation may be essential for an anticancer mechanism, especially in situations with high leptin levels, such as obesity. The tumor mechanisms of leptin involve proliferative activity in various cancer cell types, suppression of the apoptotic process, cell invasion and migration, and angiogenic modulation, in addition to affecting immune cells (Ayed et al., 2023).

ADIPOQ is expressed in adipose tissue and encodes a protein involved in metabolic and hormonal processes. Fat metabolism; insulin sensitivity; and anti-diabetic, anti-atherogenic, and anti-inflammatory actions are activities developed by this adipokine. Unlike leptin, adiponectin is present at low levels in obese individuals, due to an inflammatory profile generated by adipose tissue that hinders the secretion of adiponectin. In contrast to other low-regulated genes, this adipokine, when related to the antitumor mechanism, is the only one recognized for presenting this property through several mechanisms, mainly through the inhibitory effect observed in the PI3K/AKT/mTOR axis. Therefore, this is probably not the pathway used by organoselenium for the predicted and observed antitumor effects (Garcia-Miranda et al., 2022; Mudaliar et al., 2022).

### 3.7 Protein-Protein Interaction Network

The data were placed in the STRING database to extend and better understand the genes and interactions. The results were imported into Cytoscape to better evaluate the interactions, and a visual network was established (Figure 3). The color of the figure varies according to the number of interactions. A total of 53 targets and 512 interactions were identified. Using the Network Analyzer plug-in, the average degree value of the node was 19.32, the average Betweenness Centrality was 0.10, and Closeness Centrality was 0.67.

Figure 3 – Protein interaction network of the Organoselium's target genes



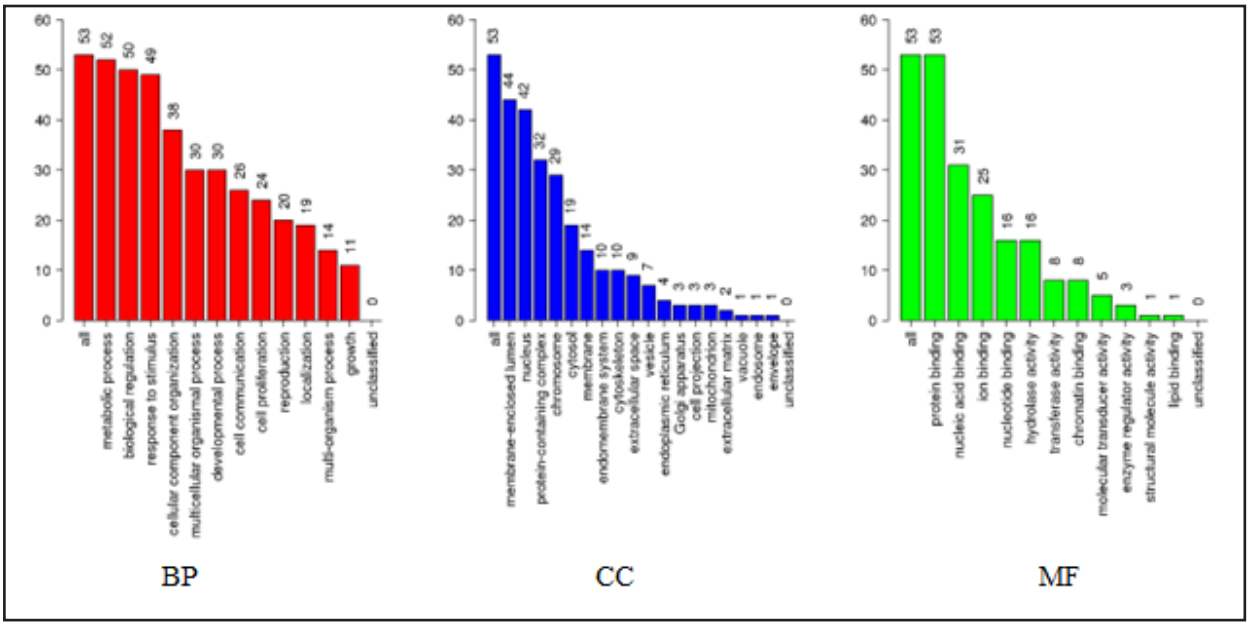
Source: Organized by the authors (2024), with data obtained by the Cytoscape platform

### 3.8 GO Enrichment Analysis

The 53 genes were uploaded to the WebGestald platform for GO enrichment analysis. Therefore, the web base allows the observation of the primary genes involved

in biological process (BP), cellular component (CC), and molecular function (MF) (Figure 4). Analysis of central BP allowed us to observe the main processes involved with the highest number of targets: metabolic process (52/53), biological regulation (50/53), response to stimulus (49/53), cellular component organization (38/53), multicellular organismal process (30/53), developmental process (30/53), cell communication (28/53), cell proliferation (24/53) and reproduction (20/53). Moreover, CC enrichment revealed the following main targets: membrane-enclosed lumen (44/53), nucleus (42/53), protein-containing complex (32/53), and chromosome (29/53). Finally, MF analysis demonstrated the following main functions: protein binding (53/53), nucleic acid binding (31/53), and ion binding (25/53).

Figure 4 – GO enrichment analysis. Biological process (BP), cellular component (CC), and molecular function (MF). The number above the bar represents the number of target genes




Source: Organized by the authors (2024), with data obtained by the WebGestald platform

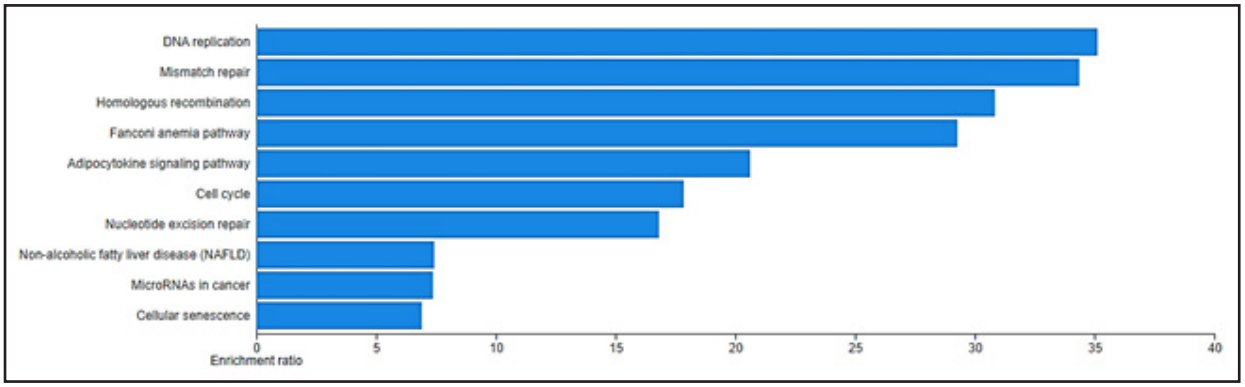
### 3.9 KEGG Enrichment Analysis

The KEEG analysis was also performed by loading the 53 genes into the WebGestalt web base, and the top 10 pathways are identified in Figure 5. The factors

found were in order of enrichment ratio: the number of observed genes divided by the expected genes in each category. Therefore, verification of the critical biological pathways involved in the genes to which AFAT-Se is possibly related is possible. The first biological pathway with the highest enrichment ratio is essential for genome stability, cell proliferation, and DNA replication (Ubhi et al., 2019). The following genes were identified: the minichromosome maintenance complex (MCM2, MCM4, MCM6, and MCM7), replication factor C (RFC3 and RFC4), and replication proteins (RPA1 and RPA2). The second point is related to the response to DNA damage that can cause cell cycle arrest and mismatch repair (Li, 2013). The genes observed were exonuclease 1 (EXO1), RFC3, RFC4, RPA1, and RPA2. Third, homologous recombination is a pathway that can repair DNA double-strand breaks (Sun et al., 2020). Thus, the group of genes involved in ATM serine/threonine kinase (ATM), BLM RecQ-like helicase (BLM), DNA repair (BRCA1 and BRCA2), RAD51 recombinase (RAD51), DNA topoisomerase II binding protein 1 (TOPBP1), and RPA1 and RPA2. The genetic disorder Fanconi Anemia appears in the fourth and is characterized by a predisposition to cancer, bone marrow failure, congenital disabilities, and difficulty in repairing cross-links in DNA chains (Niraj et al., 2019). Therefore, the verified genes were ATR serine/threonine kinase (ATR), ATR interacting protein (ATRIP), FA complementation group D2 (FANCD2), FA complementation group I (FANCI), RAD51, BLM, BRCA1, BRCA2, RPA1, and RPA2. The next point is primarily related to polypeptides secreted by adipocytes, which are linked to energy homeostasis and glucose and lipid metabolism, that is, the adipocytokine signaling pathway (Cao et al., 2022). The genes related to this pathway are ADIPOQ, LEP, adiponectin receptors (ADIPOR1 and ADIPOR2), Janus kinase 2 (JAK2), leptin receptor (LEPR), neuropeptide Y (NPY), suppressor of cytokine signaling 3 (SOCS3), and signal transducer and activator of transcription 3 (STAT3). The cell cycle parameter appears sixth, and is the machinery existing in the cell nucleus, primarily replicating genomic DNA and creating two daughter cells (Liu et al., 2019; Matthews et al., 2022). The following genes were identified in this pathway: cell division cycle (CDC25A, CDC25B, CDC25C,

CDC45, and CDC6), tumor protein p53 (TP53), origin recognition complex subunit 1 (ORC1), CHEK1, ATR, MCM2, MCM4, MCM6, and MCM7. Nucleotide excision repair is seventh in the enrichment ratio and is related to machinery capable of removing damaged DNA fragments (Kokic et al., 2019). In this way, the following genes belonging to this parameter were identified: Cullin 4A (CUL4A) and RFC3, RFC4, RPA1 and RPA2. Non-alcoholic fatty liver disease (NAFLD) is also related to the parameters found and summarizes various damage to the liver that can be caused by inflammatory processes, hepatocyte damage, and fibrosis (Pouwels et al., 2022). Therefore, the genes related to this pathology observed by the data-based tool were CCAAT enhancer binding protein alpha (CEBPA) and the genes ADIPOQ, LEP, ADIPOR1, ADIPOR2, LEPR, and SOCS3. The miRNAs in cancer factors are associated with the onset and progression of cancer, as they are expressed with changes in quality and quantity in a pathological state (Cui et al., 2019). The genes associated with this factor are, in this case, signal transducer and activator of transcription 3 (STAT3), ATM, BRCA1, CDC25A, CDC25B, CDC25C, and TP53. Cellular senescence, with a lower enrichment ratio, involves cell cycle arrest and the release of inflammatory cytokines by morphologically affected cells, presenting flattened cell bodies, vacuolation, and granularity in the cytoplasm and abnormal organelles (Huang et al., 2022). The platform indicated the following genes in this factor: HUS1 checkpoint clamp component (HUS1), RAD9 checkpoint clamp component A (RAD9A), ATM, ATR, CDC25A, CHEK1 and TP53.

Figure 5 – KEEG enrichment analysis of target genes




Source: Organized by the authors (2024), with data obtained by the WebGestald platform

## 4 DISCUSSION

In cancer therapy, multidrug resistance (MDR effect) and the absence of specificity are significant challenges inherent in traditional treatments. In this context, selenium (Se), an essential micronutrient, stands out for its biological and pharmacological properties, highlighting its potential antitumor activity. Organoselenium compounds, in particular, offer several advantages over conventional chemotherapeutic agents, starting with their potential antioxidant properties. By scavenging free radicals and reactive oxygen species (ROS), these compounds protect healthy cells from oxidative damage, which is closely associated with cancer progression. Additionally, organoselenium compounds have shown promising effects in preventing metastasis, a major challenge in cancer therapy (Gandin et al., 2018; Sak et al., 2022).

Another key benefit of the organoselenium compounds is their ability to enhance the efficacy of other cancer therapies, allowing lower doses of conventional drugs, and thus reducing toxicity. These compounds have also been shown to cause fewer adverse effects, highlighting the need for further exploration of their potential. Concerning specifically the organoselenium AFAT-Se, Leal and collaborators evaluated

its antiproliferative effects on the human bladder carcinoma cell line T24, achieving 76% growth inhibition after 48 hours of incubation (Leal et al., 2022). In the same context, a study arising from this *in silico* exploration obtained promising results using a nanoparticulate combination of the AFAT-Se with paclitaxel, demonstrating synergistic activity against resistant/MDR cancer cells in both two-dimensional and three-dimensional models. Moreover, the proposed nanosystem demonstrated good biocompatibility with healthy cells and substantial antioxidant activity, highlighting the potential of this organoselenium for further exploration as an antineoplastic agent (Mathes et al., 2024).

Drug discovery has been optimized and improved through network pharmacology and *in vitro* processes. Screening performed by these methods is paramount for discovering biological characteristics and future therapeutic efficacy (Guan et al., 2021).

Lipinski's Rule of Five relates to the drug-likeness of a compound that would be amenable to oral administration (Chagas et al., 2018). In addition to filtering in drug development, the rule of five associated with parameters such as polar surface and rotating links could interfere with the ADMET parameters. The organoselenium under analysis is consistent with Lipinski's RO5 since it only subtly violates one rule: the molecular weight (500 g/mol). However, this factor does not limit the follow-up research on this compound, considering that several antitumor drugs have even higher molecular weights (Chagas et al., 2018; Doak et al., 2014; Shultz, 2018). The log P value indicates its good lipophilicity, a parameter capable of allowing membrane transport and excellent protein binding affinity (Constantinescu et al., 2019). Another essential factor that the molecule presents is the amount of HBD (<5), considering that higher values could indicate a loss of the ability of the compound to permeate the lipid bilayer. Similarly, the HBA parameter allows for a higher acceptable number (<10), a factor wherein the organoselenium also presents a convenient result, which would also facilitate permeability (Ivanović et al., 2020).

Regarding PSA, organoselenium also fulfills the requirement of the rule (<140 Å), so it may be related to good permeability through cell membranes, as this parameter is correlated with passive transport of molecules and intestinal absorption. However, the results showed that the compound might not penetrate the blood-brain barrier (<90 Å). Associated with the PSA parameter, the rotatable links are also an ideal number in the compound, providing adequate flexibility to the compound, thus generating a greater possibility of oral bioavailability (Ivanović et al., 2020; Odi et al., 2020; Veber et al., 2002). The molecular volume parameter may intervene in the type of transport because smaller molecules are more easily diffused, whereas larger molecules have increased lipophilicity, facilitating permeation (Fong, 2015).

Pharmacokinetic properties limit the success of the drug in its journey through the body (Aouidate et al., 2018). According to the analyzed parameters, intestinal absorption was facilitated, as indicated by the three tools. Another vital aspect to consider in the phenomenon of absorption is P-gp, a recognized efflux transporter expressed in the small intestine, liver, colon, kidneys, placenta, and the blood-brain barrier, and is also involved in multidrug resistance (Zadorozhnii et al., 2022). The platforms demonstrated the inhibition of this efflux pump by the compound and its possibility to be a substrate of the same, as found by the two tools. Therefore, it is emphasized that several relevant drugs are substrates and poor inhibitors, whereas others are suitable inhibitors and median substrates of P-gp. Factors that may be involved in this aspect affect the possibility of different binding sites on the glycoprotein that define the kinetic profile and dose of the compound involved (Leopoldo et al., 2019).

In the pharmacokinetic factors related to the distribution, it can be seen that the compound could not significantly access the brain; that is, it would not be attractive for treatments with this purpose. In addition, the intermediate volume of distribution is interesting because it determines the balance between the concentration in the plasma and extravascular compartments (Korzekwa et al., 2017). High binding to proteins by the compound indicated by all platforms was also observed. Although this parameter

is essential to facilitate the transport of hydrophobic molecules in aqueous places in the human body, it may also indicate a limitation of bioavailability due to the difficulty of the compound-protein complex to cross biological membranes (Wanat, 2020).

The compound interacts with CYP3A4, possibly as a substrate and inhibitor. In the Cytochrome P450 superfamily, CYP3A4 is responsible for the metabolism of approximately 50% of clinically available drugs (Taneja et al., 2018). Being a substrate was an expected parameter because compounds with molecular weights above 360 g/mol are primarily metabolized by this CYP, thus aiding compound clearance (Hu et al., 2020). However, owing to the complex interaction between CYP3A4 and its substrate, inhibitor, or inducer, AFAT-Se also has CYP3A4 inhibitory properties according to the platforms used. It is also worth highlighting that this CYP has active sites that allow the accommodation of substrate and inhibitor simultaneously and that further studies are necessary to define the specific conditions and dosage required for these phenomena (Kondza et al., 2021; Wright et al., 2019). As the molecule is a CYP2D6 substrate, metabolism could be facilitated, as this enzyme is responsible for approximately 20-30% of commonly prescribed drugs (Lee et al., 2021).

When evaluating renal elimination, the compound does not appear to be a substrate of OCT2 but possibly an inhibitor, which may hinder excretion via this route (George et al., 2021). However, the total clearance is perhaps high, which could mean good elimination from the blood in a short period. This may be related to compensating factors, hepatic metabolism, dosage, safe therapeutic window, and same-drug interactions, considering that this is a global estimate of elimination efficiency that does not define where and how it occurs. This complex interaction depends on the clinical context and characteristics of the AFAT-se compound (Rowland et al., 2022).

Toxicity parameters such as MTD are essential for the initiation and follow-up of studies. Furthermore, hERG inhibition must be evaluated carefully because while it may cause a delay in repolarization and impair cardiac potential, it may also be responsible for exciting action, if selective, in the context of a therapeutic target in

cancer cells (Garrido et al., 2020; He et al., 2020). When evaluated using the Ames test, it was observed that AFAT-Se might not cause genetic mutations. Therefore, in theory, the compound does not act as a carcinogen because many carcinogens are mutagens (Zeiger, 2019). Another critical point is the inclusion of LD50 in the moderate toxicity range, given the relatively high value found, which indicates that a substantial amount of the substance is necessary to cause death in 50% of the test animals. In addition to these factors, the LOAEL value was also satisfactory and may indicate good performance in a chronic context (Raies et al., 2016).

The possible hepatotoxicity demonstrated by these platforms also requires further studies, as the extent of the influence on the liver is not considered and may depend on the clinical context of use (Real et al., 2019). However, skin sensitization and nephrotoxicity may not appear as problems in subsequent studies. The results were evaluated in *Tetrahymena pyriformis*, a protozoan used in ecotoxicology studies, and in minnow fish, an essential indicator of aquatic ecosystems, signal positive values, which suggests that the substance may be harmful to these organisms and the marine environment (Acosta et al., 2020). However, complete interpretation requires additional analysis of real exposure but signals the need for further study of this context.

Concerning biological activity, the importance of a molecule's potential in cancer treatment can be seen. The ability to be antineoplastic highlights the ability to inhibit the abnormal growth of benign or malignant cells or even their dissemination. Added to this factor is the possibility of improving the expression of TP53, which limits the increase in the maintenance of DNA integrity and prevents the proliferation of damaged cells (Williams et al., 2016). Likewise, being anticarcinogenic introduces the possibility of cancer prevention associated with chemoprotection, which would help the body defend against harmful agents (Anisimov et al., 2012).

The AFAT-Se compound showed evident cytotoxicity in the tested cell line, as detected by the MTT assay, more prominently at the highest concentrations tested. The cell line used in the study, HT-29, was one of those found by the CLC-Pred web-based

and corroborated the indication that the compound presents activities evidenced by the WAY2Drug platform (Druzhilovskiy et al., 2017; Lagunin et al., 2023). Therefore, possible targets must be identified for the discovery of molecules that can become pharmaceuticals. The relationships among drugs, genes, and proteins are complex, and initial *in silico* analysis can clarify this context. The first and foremost possible targets that might trigger the action of AFAT-Se are listed in Table 2. In addition to these genes, the String platform allowed us to expand this understanding, identify some interaction measures, and show additional targets that can be influenced by the compound (Szklarczyk et al., 2023).

Additional GO and KEEG analyses made it possible to group genes that influenced specific processes and allowed for a greater understanding of the action of these targets. When observing the results found in the GO analysis for BP items, one can perceive the influence on regulatory processes such as metabolic processes, biological regulation, response to stimulus, cellular component organization, multicellular organismal process, developmental process, cell communication, cell proliferation, and reproduction, which are closely linked to antitumor effects. Combined with these results, the KEEG analysis continues to agree with this fact, identifying interference in DNA replication, mismatch repair, homologous recombination, and cell cycle, among others of interest for future studies on the possible antitumor action of the AFAT-Se compound (Guan et al., 2021).

## 5 CONCLUSIONS

In this *in silico* and *in vitro* study, the organoselenium compound AFAT-Se appears as an exciting molecule, particularly in cancer therapy. It demonstrated promising drug-like properties with favorable pharmacokinetic characteristics that suggest good oral bioavailability and potential for intestinal absorption. AFAT-Se also displayed notable cytotoxic effects toward the HT-29 tumor cell line, evidencing its potential as an antineoplastic agent. Therefore, this study contributes to the ongoing

search for effective cancer therapies by investigating the antitumor potential of an innovative organoselenium compound. Our combined *in silico* and *in vitro* approaches identified critical molecular targets within cancer-related pathways and provided a comprehensive assessment of AFAT-Se's ADMET properties and anticancer activity. The observed encouraging pharmacokinetic profile and cytotoxicity against a human colorectal tumor cell line offer compelling evidence supporting further preclinical investigation of AFAT-Se as a potential therapeutic agent for cancer.

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